ELISA -KIT FOR DIAGNOSIS OF SHEEP AND GOATS PARATUBERCULOSIS

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Summary

A LAM -antigen from culture of *Mycobacterium paratuberculosis* was prepared and used for antibody capture in an ELISA kit for the diagnosis of sheep and goat paratuberculosis. The kit was checked using positive and negative sera tested by ELISA using a commercial kit, registered in Romania, as reference. The results indicated a good kit behavior and agreed with those for the reference kit as to sensitivity and specificity. The kit was registered as ELIPARA-OC.

Key words: LAM- antigen, ELISA, paratuberculosis, diagnosis, sheep, goat.

Paratuberculosis (Johne's disease) is a chronic granulomatous enteritis of ruminants caused by *Mycobacterium avium ssp. paratuberculosis (M. paratuberculosis).* (2). The disease is present in all continents, different countries applying different control measures (6).

In small ruminants, the only consistent, though nonspecific, clinical sign of the disease is progressive weight loss. Diarrhea is not a constant feature and is often intermittent, although it can be severe in some animals in individual herds (4). No efficient treatment is available. The vaccination is currently not used.

In animals with clinical signs suggesting Johne's disease, agar gel immunodiffusion (AGIT) might be a highly sensitive and specific test (8).

However, the detection of subclinical cases is considered as the critical step in the control of the disease.

The enzyme-linked immunosorbent assay (ELISA) is the current method used in serologic diagnosis of paratuberculosis. Improvements of performances by pre-absorbing the test sera with a suspension of *M. phlei* (10) and using an affinity-purified peptide antigen were reported (1). Also, higher sensitivity of ELISA using the lipoarabinomannan (LAM), a non-protein antigen, when diagnosing paratuberculosis in sheep was observed (9).

An ELISA kit with LAM- type antigen, developed by us for serologic diagnosis in bovine paratuberculosis, was previously reported (7).

In this paper, an ELISA kit with LAM- type antigen from *M. paratuberculosis*, also including pre-absorbtion of sera with *M. phlei*, for serologic diagnosis in sheep and goat paratuberculosis, is presented. In a comparative study on goat paratuberculosis diagnosis, experimental ELISA with same antigen showed good sensitivity and specificity (3).

Materials and methods

Antigen The antigen is prepared from 10±1 weeks- old culture (liquid Reid medium) of *M. paratuberculosis,* strain 8578 (Weybridge, UK).The preparation scheme, adapted from technique described by Jark et al,1997 (7), consisted of the following steps: the collection of wet bacterial mass, heat inactivation, disruption of bacilli by ultasonication, clarifying by double centrifugation, protein digestion with proteinase K, clarifying by centrifugation, purified and concentrated by centrifugal filtration; preserved with thiomersal, stored at -20^oC.

Kit components

LAM- antigen coated micro- plates*:* the antigen is diluted in citrate buffer, pH 6 and then placed in the wells of the micro- plate. (Nunc MaxiSorp). The plates are incubated at 4^oC for 16 hours, then washed, stabilised and dried in warm air.

Positive control serum (PCS): collected from sheep and goats with anti- *M. paratuberculosis* antibodies developed by immunization, tested by AGIT, CF and ELISA, then filtered and preserved with thiomersal.

Negative control serum (NCS*):* collected from sheep and goats in paratuberculosis- free herds, tested by AGIT, CF and ELISA, then filtered and preserved with thiomersal.

Anti- sheep/ goat immunoenzymatic conjugate (x 100): It consists of anti-*sheep/ goat* IgG coupled with peroxidase (Sigma).

The working dilutions of the immunoactive components are determined by chessboard ELISA titration.

Substrate**:** solution of H_2O_2 in citric buffer, pH 4.

Chromogen**:** ABTS [2,2' –azino-bis (3-ethylbenzthiazoline)-6-sulphonic acid] - solution in distilled water.

Sera and conjugate diluent: phosphate buffer saline (PBS) with bovine serum albumin (Merck), $Tween_{20,}$ *M.phlei-* antigen and thiomersal.

Washing solution (x10): PBS, with $Tween_{20}$ and thiomersal.

Stop reagent**:** 1.5% solution of NaF in distilled water.

ELISA

Use is made of the indirect variant with capture antigen. The steps are as follows:

Reagents thermal balancing**:** The kit components, with the exception of the conjugate 100x, are brought to the room temperature 30 minutes at least before use under gentle agitation for homogenization.

Control sera dilution**:** 1/20 in the serum and conjugate diluent.

Samples dilution**:** 1/200 in the serum and conjugate diluent

Sera distribution into the plates wells: 100 μl/well of the positive control serum (2 wells), the negative control serum (2 wells) and the test sera (92 samples).

Incubation: at 37^oC, for 15 minutes, under agitation.

Plate washing**:** four times.

Diluted conjugate distribution**:** 100 μl/ well.

Incubation: at 37°C, for 15 minutes, under agitation.
Plate washing: four times.
Substrate + chromogen (11 ml + 0.45 ml) mixture distribution: 100 μl/ well.
The plate is left at room temperature protected from light.
Plate reading: after 30 minutes, by means of a multi-channel photometer at 405 nm, and:
- if the validation parameters are reached, the reaction is stopped immediately with 50 μl/well of the stop solution;
- if the validation parameters are not reached, the plate is left standing up to 45 minutes and then stopped as above with final OD values reading.

Reaction validation parameters
- the OD (mean value) of the negative control serum: lower than 0.350;
- the OD (mean value) of the positive control serum: higher than 1.000;

Results calculation

The samples which OD values are equal or smaller than OD mean value of negative control + 0.200 are considered as negative (animals non- infected)
The samples which OD values are higher than OD mean value of negative control + 0.200 and smaller than OD mean value of negative control + 0.300 are considered as doubtful (animals must be re-tested*)*
The samples which OD values are higher OD mean value of negative control + 0.300 are considered as positive (animals infected with *M. paratuberculosis)*

Results and discussions

The kit performances were determined by testing with positive and negative sera examined by ELISA performed with a kit registered in Romania.

Detectability

Positive control serum (PCS): 1/1 and 1/2, 1/4, 1/8 and 1/16 dilutions in negative serum, two wells each.
Negative control serum (NCS): 1/1, two wells.
Reaction conditions: as per the technique described.
Results:

Positive control serum	Final dilution	1/200	1/400	1/800	1/1600	1/3200
	O.D.	1.672	0.1120	0.727	0.461	0.364
Negative control serum	O.D.	0.183	0.183	0.183	0.183	0.183
	Final dilution	1/200	1/200	1/200	1/200	1/200

Conclusion:
Positive control serum reached OD value of negative control serum at a final dilution higher as 1/3200 (minimum 16 time higher as work dilution).

Repeatability

Inter-wells variability

Positive control serum (PCS)

Negative control serum (NCS)

Four wells each. in three areas of the plate

Reaction conditions: as per the technique described

Results:

	1	*2*	*3*	*4*
A	2.406	2.367	2.298	2.375
B	0.184	0.203	0.256	0.265
	5	*6*	*7*	*8*
D	2.454	2.441	2.467	2.510
E	0.265	0.281	0.277	0.265
	9	*10*	*11*	*12*
G	2.414	2.453	2.481	2.113
H	0.272	0.260	0.247	0.198

Conclusion: Variability coefficients (standard deviation/mean value):

PCS = 0.0445 NCS = 0.1338

Inter-plates variability

PCS

NCS

Five plates, four wells/serum/plate

Reaction conditions: as per the technique described

Results:

		5	*6*	*7*	*8*		*5*	*6*	*7*	*8*
pl. 1	D	2.441	2.441	2.467	2.510	E	0.265	0.281	0.277	0.265
pl. 2	D	2.452	2.465	2.508	2.365	E	0.276	0.300	0.284	0.274
pl. 3	D	2.450	2.450	2.463	2.389	E	0.332	0.328	0.350	0.317
pl. 4	D	2.462	2.490	2.436	1.250	E	0.313	0.300	0.263	0.297
pl. 5	F	2.426	2.406	2.493	2.163	G	0.285	0.281	0.271	0.256

Conclusion: Variability coefficients (standard deviation/mean value):

PCS = 0.0330 NCS = 0.0889

Sensitivity

21 ELISA- positive∗ sheep sera (natural infection)

9 ELISA- positive∗ goat sera (natural infection)

(∗ELISA kit, registered in Romania)

PCS

NCS

Reaction conditions: as per the technique described

Results:

		ELIPARA-OC		
		P	D	N
Sheep positive sera	21	21	0	0
Goat positive sera	9	9	0	0

Conclusion: all samples are positive.

Specificity
59 ELISA- negative∗ sheep sera
37 ELISA- negative∗ goat sera
(∗ELISA kit, registered in Romania)
PCS
NCS
Reaction conditions: as per the technique described
Results:

		ELIPARA-OC		
		P	D	N
Sheep negative sera	59	0	0	59
Goat negative sera	37	0	0	37

Conclusion: all samples are negative.

Validity
Coated plate, stored at 2-8°C for 12 months
PCS/4 wells
NCS/4 wells
Reaction conditions: as per the technique described
Results:

	1	*2*	*3*	*4*
C	1,278	1,236	1,231	1,306
D	0,294	0,296	0,293	0,295

Conclusion:

The validation parameters are reached under the afore-mentioned experimental conditions, the performances of this ELISA kit are acceptable and concordant with the reference one as far as its sensitivity and specificity are concerned; the kit was, therefore, proposed and accepted for its registration as ELIPARA-OC.

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